ABSTRACT

OBJECTIVE: To describe the investigation of a sylvatic yellow fever outbreak in the state of Sao Paulo and the main control measures undertaken.

METHODS: This is a descriptive study of a sylvatic yellow fever outbreak in the Southwestern region of the state from February to April 2009. Suspected and confirmed cases in humans and in non-human primates were evaluated. Entomological investigation in sylvatic environment involved capture at ground level and in the tree canopy to identify species and detect natural infections. Control measures were performed in urban areas to control Aedes aegypti. Vaccination was directed at residents living in areas with confirmed viral circulation and also at nearby cities according to national recommendation.

RESULTS: Twenty-eight human cases were confirmed (39.3% case fatality rate) in rural areas of Sarutaiá, Piraju, Tejupá, Avaré and Buri. The deaths of 56 non-human primates were also reported, 91.4% were Alouatta sp. Epizootics was confirmed in two non-human primates in the cities of Itapetininga and Buri. A total of 1,782 mosquitoes were collected, including Haemagogus leucocelaenus, Hg. janthinomys/capricornii, and Sabethes chloropterus, Sa. purpureus and Sa. undosus. Yellow fever virus was isolated from a group of Hg. Leucocelaenus from Buri. Vaccination was carried out in 49 cities, with a total of 1,018,705 doses. Nine serious post-vaccination adverse events were reported.

CONCLUSIONS: The cases occurred between February and April 2009 in areas with no recorded yellow fever virus circulation in over 60 years. The outbreak region occurred outside the original recommended vaccination area with a high percentage of susceptible population. The fast adoption of control measures interrupted the human transmission within a month and the confirmation of viral circulation in humans, monkeys and mosquitoes. The results allowed the identification of new areas of viral circulation but further studies are required to clarify the dynamics of the spread of this disease.

INTRODUCTION

Yellow fever (YF) is an infectious disease, endemic in the rainforests of Latin America and Africa, provoking outbreaks and epidemics which impact on public health. The disease is caused by a virus from the Flaviviridae family, genus Flavivirus, and transmitted by the bite of bloodsucking insects of the Culicidae family, genus Haemagogus and Aedes. Urban and sylvatic cycles are how it is classically described. Sylvatic yellow fever (SYF) occurs in forested areas, where it is transmitted by mosquitoes to non-human primates (NHP), amplifying hosts of the virus. Occasionally, susceptible humans who enter in contact with forested areas become infected. The clinical manifestation of the disease can be asymptomatic, or with mild or moderate symptoms, or serious and malignant. Mortality varies between 5.0% and 10.0%, reaching 60.0% in the most severe forms.

According to the World Health Organization, YF is endemic in 33 countries in Africa, with 23 at high risk of epidemic. Although a highly effective vaccine is available, epidemiological data show a resurgence of areas in which the virus circulates in West Africa and the Americas over the last 20 years.

The last report of urban transmission in Brazil occurred in the state of Acre, Northern, in 1942. From this time onwards, only sporadic sylvatic transmission occur in endemic area, with records of cyclical epidemics at regular intervals of five to seven years, alternating with small numbers of cases, a pattern observed until 1997.

From 1998 onwards, a change in this pattern was noted, with increased number of cases and expansion in the disease transmission area, cases in regions that had been previously unaffected and outbreaks in the states of Pará and Tocantins and in the Chapada dos Veadeiros National Park in Goiás, Northern Brazil, in 2000. Since then, there have been reports of transmission in the states of Acre, Amazonas, Pará, Tocantins, in region Northern Brazil, and Bahia, Northeastern Brazil, Minas Gerais, Southeastern Brazil, Mato Grosso, and Goiás, and in the Federal District, in Region Midwest Brazil.

Occurrence of sylvatic yellow fever (SYF) increased in Brazil in 2008, based on records of deaths in non-human primates and cases in humans in the states of Sao Paulo, Rio Grande do Sul, Penapolis, Tanabi, Mirassol and Monte Aprazível. In 1936, there was a record of epidemics in municipalities in the Alta Sorocaba region, especially in the cities of Avaré, Piraju, Assis and Itapeva, with the last recorded outbreak occurring in 1953. After almost 50 years with no reported epidemics, two autochthonous cases of SYF were confirmed in the municipalities of Santa Albertina and Ouroeste, in 2000, and a suspected epizooty in Miguelópolis, in 2003. In 2008, virus circulation was confirmed in the state, with an epizooty in the municipalities of Mendonça, Nova Aliança and Urupês and two autochthonous human cases in Luiz Antonio and Sao Carlos. The spread of the virus resulted in a widening of the areas in which vaccination is recommended for residents and visitors.

In February 2009, SYF transmission was detected in the region of Botucatu, a non-endemic area where vaccination has not been recommended. The Ministry of Health considered the event to be a public health emergency of national importance.

The aim of this study was to describe the investigation of an outbreak of sylvatic yellow fever in the state of Sao Paulo and the principle control measures utilized.

METHODS

A descriptive study of an outbreak of SYF in 2009, covering the southeast of the state of Sao Paulo, encompassing 49 municipalities, with an estimated population of 1,174,142 inhabitants. The majority of the municipalities in this region (more than 70.0%) belong to the Alto Paranaíba basin, with an area of 28,447 km². The study took place between February and April 2009.

For case definition, the Ministry of Health recommendations in the epidemiological vigilance guidelines were used. Individuals who had not been vaccinated, who had a high fever accompanied by jaundice and/or hemorrhage, resident or having come from an SYF risk area in the preceding 15 days were considered to be suspected cases.

After the virus circulation has been confirmed, the most sensitive case definition was employed: individuals who had not been vaccinated, with a high fever (up to seven days) accompanied by at least two symptoms (headache, myalgia, nausea or vomiting), resident or having come from an SYF risk area in the preceding 15 days were considered to be suspected cases.
retrospective assessment of deaths from unknown causes and/or from icteric-hemorrhagic syndrome.\textsuperscript{d}

Suspected cases that had reactive results in at least one diagnostic method (detection of IgM antibodies using the MAC-ELISA technique, virus isolation, detection of viral genome or viral antigen using the immunohistochemistry test) and with a clinical-epidemiological link (suspected death without samples being collected in the area of transmission during the period) were considered confirmed cases.\textsuperscript{d}

A serological survey was applied between March 16 and 19 in the municipality of Sarutaiá, where there were no reports of human cases or epizooty (not considered at risk of the disease) and where the first confirmed case resided. The individual had not been vaccinated for yellow fever and the only place he visited was a leisure area within the municipality. The aim of the survey was to detect circulation of the virus in the municipality and identify other cases. To calculate the sample size, an expected disease frequency of 50.0% was assumed, with level of significance of 95.0% and acceptable error of 10.0%. For a population of 2,966 inhabitants, with 996 residences divided into 9 sectors covered by the Family Health Care Program, the houses were randomly selected and 93 individuals were included in the study. Blood samples from 86 asymptomatic participants were collected for serology (Mac Elisa IgM).

Deaths of NHP with laboratory evidence of the virus infection in at least one animal was considered to be epizooty of yellow fever.\textsuperscript{d}

The data on humans and NHP were taken from Information System for Notifiable Diseases (SINAN) forms and standardized spreadsheets. Notifications were made to the State Epidemiological Health Department by telephone, fax or electronically. Probable locations of infections were identified and georeferenced using Garmin model Etrex (GPS). The geographical coordinates were obtained and referred to the Datum SAD 69. The municipalities were mapped using the Brazilian Institute of Geography and Statistics (IBGE) 2007 Digital Municipal Grid and the Mapinfo program, version 7.0, Mapinfo Corporation.

The blood, serum and cerebrospinal fluid samples were processed using the Mac-Elisa test, following the protocol described by Kuno et al.\textsuperscript{13}

Samples of human blood and serum, tissue material suspensions obtained from NHP autopsies and mosquitoes were inoculated in 1 to 3-day-old Swiss mice. The brains of animals that showed signs of the disease underwent the following steps.\textsuperscript{13} The samples were inoculated in cell cultures of mosquitoes \textit{Ae. albopictus}, clone C6/36.\textsuperscript{10} Identification of the isolates was proceeded using the indirect immunofluorescence technique standardized by Gubler et al.,\textsuperscript{3} using a polyclonal anti-flavivirus. Positive samples were typed by indirect immunofluorescence with monoclonal antibodies for the yellow fever virus YFV (Biomanguinhos – RJ).

**Polymerase chain reaction:** The extraction was performed from total RNA from tissue samples, serum or suspension prepared from mice previously inoculated with the use of specific commercial kits: QIAamp\textsuperscript{®} RNA Blood for tissues and QIAamp\textsuperscript{®} Viral RNA Kit for serum (Qiagen Inc., Ontário, Canadá), following the manufacturer’s instructions. Amplification of viral RNA was performed by single-step reverse transcriptase (one step RT-PCR) followed by a second amplification (semi-nested).\textsuperscript{6} The amplified products were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide to verify band size.

**DNA sequencing:** ABI-377 sequencer was used to sequence positive samples and Chromas software version 1.45 and EditSeq (Lasergene DNASTAR Inc.) for editing nucleotide sequence.

**Histopathology and immunohistochemistry:** Samples of brain, heart, lung, liver, spleen and kidney, fixed in formaldehyde and embedded in paraffin were processed and examined after completion of histological sections and staining with hematoxylin and eosin. Slices of liver with 0.3 µm were placed on slides with silane and subjected to immunohistochemistry with polyclonal anti-yellow fever virus, diluted 1/2,000 and detection system using mouse and rabbit anti-immunoglobulins combined with peroxidase or alkaline phosphatase (Envision\textsuperscript{®}, Dako Cytomation, EUA).\textsuperscript{9}

Entomological investigation activities were triggered by notification of the death of a NHP and suspected and/or confirmed cases of yellow fever in humans.

Capture took place at ground level and in the tree canopy, on the edges and within the forests at locations of probable infection (LPI). The mosquitoes were collected using protected humans with mouth aspirators and nets at 15 to 20 minute intervals between 9:00 am and 4:00 pm, for four days in each area. Center for Disease Control and Prevention (CDC) style dry ice traps were also used.\textsuperscript{7} In order to try and record natural infection the mosquitoes were stored in cryo-resistant tubes transported in liquid nitrogen and then frozen and stored at -70°C for later identification and grouped into batches and processed for virus isolation.

Vector control measures in urban areas of municipalities infested with \textit{Ae. aegypti} were developed for the immature (control breeding) and adult (spraying) stages in areas where suspected and or confirmed cases were

resident or had visited, according to the current tech-
nological norms.8 Before these actions came into force,adult mosquitoes were captured for virus isolation using
aspirators and traps.

Entomological monitoring was intensified in municipalities
not infested with Ae. aegypti to research the larvae, elimi-
nate potential breeding sites and find adult mosquitoes.

Vaccination for yellow fever, 17DD Biomanguinhos,
was recommended according to the Brazilian Ministry
of Health epidemiological vigilance guidelines,d which,
in case of epidemics, include individuals aged over
six months resident in municipalities with confirmed
circulation of the virus and neighboring municipalities.
Vaccination was given house to house in the rural areas
and in health care centers in the urban areas.

Cases of serious adverse events after vaccination were
classified as acute viscerotropic disease (AVD) and acute
neurotropic disease according to CDC definitions.4

RESULTS

There were 138 reported suspected human cases
in the region studied between February and April
2009. Among them, 110 were excluded and 28 were
confirmed, of which 11 resulted in death (mortality
39.3%). The probable locations of infection in these
confirmed cases were rural areas in the municipalities
of Sarutaiá, Piraju, Tejupá, Avaré and Buri (Table 1).
Ten cases were in rural workers who worked in the
forest and 18 in individuals who undertook some kind
of leisure activity outdoors.

Eighteen of the 28 confirmed cases were male (64.3%).
Age varied between three days old and 52 years old (mean
29 years). Four cases occurred in children aged between
three days and 16 years. The confirmed cases had mild,
moderate and serious symptoms; of the confirmed cases,
50.0% fulfilled the criteria of fever, jaundice and/or hemor-
rhage, and 71.0% were hospitalized. With regards to the
laboratory diagnosis, 42.9% of cases were confirmed using
serology and 50.0% by more than one method (Table 2).

No yellow fever antibodies were detected in a serological
survey carried out on an asymptomatic sample of the
population in the municipality of Sarutaiá.

The first confirmed case occurred in the municipality
of Sarutaiá, with symptoms beginning on February
22 and the last case was in the municipality of Buri on
April 1 (Figure 1).

Histopathological findings, identified in five cases,
consisted of predominantly mediozona lesions,
extending to the hepatic parenchyma, the presence of
apoptosis and necrotic foci, micro and macrogroticular
steatosis, hyperplasia and hypertrophy of Kupffer cells
and portal spaces with slight lymphoid infiltration, with
no signs of lesion to the interface. There was positive
immunostaining for YF antigens in hepatocytes and
Kupffer cells in immunohistochemistry.

There were 56 reported NHP deaths, followed up until
August, distributed throughout seven municipalities
in the region, and 91.4% of the NHP were
Alouatta sp genus. The highest number of NHP deaths (77.5%
of the total) were reported in the municipality of Buri.
Material for laboratory diagnosis was collected from
7.0% of the animals, with viscera, serum/blood or brains
collected. Laboratory epizooty was confirmed in two
NHP of the Alouatta sp genus; one in the municipality
of Buri using the RT-PCR technique on serum and the
other was in Itapetininga using immunohistochemical
technique on the viscera. The Histopathological find-
ings were similar to those of the humans.

Entomological activities began in Sarutaiá, Piraju and
Itatinga immediately after confirmation of the first
cases, and later in Avaré, Buri and Itapetininga. There

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Table 1. Distribution of confirmed and suspected cases of sylvatic yellow fever in humans, according to probable municipality of infection. Sao Paulo State, February to April, 2009.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Confirmed</th>
<th></th>
<th></th>
<th></th>
<th>Suspected</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>%</td>
<td>Deaths</td>
<td>%</td>
<td>Cases</td>
<td>%</td>
<td>Deaths</td>
</tr>
<tr>
<td>Avaré</td>
<td>4</td>
<td>14.3</td>
<td>1</td>
<td>9.1</td>
<td>11</td>
<td>8.0</td>
<td>2</td>
</tr>
<tr>
<td>Buri</td>
<td>5</td>
<td>17.8</td>
<td>3</td>
<td>27.3</td>
<td>8</td>
<td>5.8</td>
<td>3</td>
</tr>
<tr>
<td>Piraju</td>
<td>11</td>
<td>39.3</td>
<td>5</td>
<td>45.4</td>
<td>64</td>
<td>46.4</td>
<td>7</td>
</tr>
<tr>
<td>Sarutaiá</td>
<td>7</td>
<td>25.0</td>
<td>2</td>
<td>18.2</td>
<td>53</td>
<td>38.4</td>
<td>3</td>
</tr>
<tr>
<td>Tejupá</td>
<td>1</td>
<td>3.6</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>1.4</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>100.0</td>
<td>11</td>
<td>100.0</td>
<td>138</td>
<td>100.0</td>
<td>15</td>
</tr>
</tbody>
</table>

Sources: Sistema de Informação de Agravos de Notificação. Divisão de Zoonoses do Centro de Vigilância Epidemiológica.

a Included only municipalities with confirmed circulation of yellow fever.
b Deaths are included in total cases.

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were infestations of Ae. aegypti in homes in Piraju and Avaré, while no infestations were found in Sarutaiá, Tejupá, Itatinga or Buri. Activities to control the immature and adult forms of Ae. aegypti were performed in infested municipalities. Entomological control measures were carried out in all homes in Piraju, where the highest concentration of suspected and confirmed cases occurred, spraying homes with an adulticide over a ten day period (Table 3). No investigation was conducted in Tejupá, which was included as a transmission area after a retrospective assessment.

There were 1,782 female specimens from 58 species of mosquitoes collected in the forest during the entomological monitoring activities. Among the groups identified, the following stood out: Ae. serratus group (32.2%), Psorophora ferox (22.4%), Hg. leucocelaenus (5.5%), Ochlerotatus scapularis (3.0%), Hg. janthinomys/capricornii (2.2%) and Ae. albopictus (0.9%). Specimens from the Sabethini (11.9%) genus were also found: Sa. purpureus, Sa. chloropterus, Sa. undosus, Sa. intermedius, Sa. albiprivus and Sa. tridentatus.

Among the mosquitoes captured for virus isolation, 1,782 were processed in 281 batches, constituted according to species, location and time of capture, with 58 species identified. There were 1,210 specimens, 26 batches, captured in the municipality of Buri during the first fortnight of April. The YF virus was isolated in mice and in cells, identified using RT-PCR, from a batch of the Hg. leucocelaenus species, composed of six specimens.

### Table 2. Confirmatory criteria of the human cases of sylvatic yellow fever. Sao Paulo State, 2009.

<table>
<thead>
<tr>
<th>Confirmatory criteria</th>
<th>Diagnostic method</th>
<th>no. of confirmed cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratorial</td>
<td>Serology</td>
<td>12</td>
<td>42.8</td>
</tr>
<tr>
<td></td>
<td>Serology and RT-PCR</td>
<td>8</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>Serology, RT-PCR and viral isolation</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>Serology, RT-PCR, viral isolation and Immunohistochemistry</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>RT-PCR, viral isolation and Immunohistochemistry</td>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td>Clinical epidemiological</td>
<td>Not done</td>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>28</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>


RT-PCR: polymerase chain reaction


**Figure 1.** Temporal distribution of human cases of yellow fever, according to municipality probable date of infection and onset of symptoms. Sao Paulo State, February-April, 2009.
Table 3. Distribution of the number of properties covered in the control of *Aedes aegypti* in municipalities with circulation of the Yellow Fever virus, according to type of activity and who carried it out. Sao Paulo State, March-April, 2009.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>SUCEN</th>
<th>Spraying</th>
<th>Control breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avaré</td>
<td>2,920</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buri</td>
<td>4,420</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Itapetininga</td>
<td>1,705</td>
<td>0</td>
<td>526</td>
</tr>
<tr>
<td>Itatinga</td>
<td>241</td>
<td>3,223</td>
<td>0</td>
</tr>
<tr>
<td>Piraju</td>
<td>11,724</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sarutaiá</td>
<td>339</td>
<td>1,066</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>21,349</td>
<td>4,289</td>
<td>526</td>
</tr>
</tbody>
</table>

Source: Superintendência de Controle de Endemias da Secretaria de Estado da Saúde de São Paulo (SUCEN).

Figure 2 shows the YF transmission areas confirmed in laboratory in humans, NHP and mosquitoes.

Vaccination was initiated immediately following confirmation of the first case, in Sarutaiá, and was rolled out into areas of probable viral circulation. There were 49 municipalities where vaccination was recommended, with a general population of 1,174,142. A total of 1,018,705 doses of vaccine were given between March and April, with vaccination coverage of 86.8%. There were three confirmed cases of acute neurotropic disease, one case of immediate hypersensitivity, all of whom recovered, and five cases of acute viscerotropic disease, who died. The recent circulation of the yellow fever virus in Avaré, Buri, Itapetininga, Piraju, Sarutaiá and Tejupá in the state of Sao Paulo lead to the recommended area of vaccination being expanded.

**DISCUSSION**

Confirmed cases of SYF occurred between February and April 2009 in the Southeast of the state, in areas where there had been no virus circulation recorded in more than 60 years and, therefore, not recommended for yellow fever vaccine and with a susceptible human population. The region is mountainous with a large number of
rivers and fast flowing streams. The vegetation consists of small, broken up forest formations interspersed with plantations, pasture and areas of reforestation, with favorable conditions for viral circulation.

The cases either lived or travelled through the forested area for leisure activities or work in the municipalities of Sarutaiá, Piraju, Tejupá, Avaré and Buri.

The swiftness of the joint and integrated activities carried out by the diverse organizations was essential in confirming viral circulation and adopting appropriate control measures, interrupting human transmission within a month. The vaccination campaign was started in 49 municipalities immediately after confirmation of the first human case. More than a million individuals were vaccinated in three months. The vaccination activities were expanded gradually in the municipalities according to the LPI of confirmed cases: first, in every house in the rural area and later in health facilities in urban area. Vaccination was recommended for travelers heading to the affected area and no imported cases were detected in this period.

The investigation of suspected cases by monitoring acute icterohemorrhagic febrile syndrome and deaths from unknown causes was an important instrument in identifying the first cases. Using the more sensitive definition for suspected cases throughout the work allowed mild and moderate cases to be identified.

Human cases without early detection and reporting epizooty shows the need to intensify epizootic vigilance so that it constitutes a sentinel event of the virus circulation. Even after the active investigation, no relevant monkey deaths were verified, except in Buri, in contrast to Rio Grande do Sul. In Buri, it was possible to identify the virus in humans, NHP and in mosquitoes, which made it possible to complete the epidemiological chain associated with this form of the disease.

The hypothesis of urban transmission of YF was raised in Piraju due to the large number of human cases in an area with *Ae. aegypti*. The epidemiological investigation, however, confirmed that those cases had conducted activities in the forested area near their homes where entomological research confirmed the presence of sylvatic vectors. This made the possibility of urban transmission less likely and confirmed the disease’s sylvatic transmission.

Identification of transmission in forested areas highlights the risk of infection for the human population living close by or travelling through these areas, or other similar areas in the state.

In most investigated locations, *Hg. leucocelaenus* was more common than *Hg. janthinomys/capricornii*. This created a doubt as to their role as the primary or secondary vector in the transmission of sylvatic yellow fever. *Hg. leucocelaenus* was described as an abundant species in the South of the country. Recent epizooties in Rio Grande do Sul have raised the hypothesis that this species may be a primary vector. *Sa. chloropterus* is also considered a secondary vector, as isolation has been done in naturally infected specimens. Among the mosquitoes captured, *Oc. scapularis* and *Ps. ferox* were the species with proven experimental transmission and positive for viral isolation. In the state of Sao Paulo, the virus was only isolated in *Hg. leucocelaenus* in Buri.

In addition to confirming circulation of the yellow fever virus in this area, it is important that investigation continues in order to understand the dynamics of viral transmission. Initial questioning raises the doubt as to whether the disease could have been circulating in this area, undetected, for years. The possibility should also be considered that the virus was recently introduced. Possible routes include from the state of Paraná, with records of NHP in the border area the previous year, or introduction into the state of Sao Paulo by humans due to population movement in the viremic period, trafficked NHP or some other vector. Other possible routes should not be ruled out. The use of molecular techniques such as sequencing and phylogenetic analysis of the virus may contribute to the knowledge of circulation and viral origin in this region.

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Reemerging of Yellow fever in Sao Paulo

Mascheretti M et al

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The authors declare that there are no conflicts of interest.